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EPIDEMIOLOGICAL DESCRIPTION OF A NEW VIRAL DISEASE AFFLICTING CULTURED *CYPRINUS CARPIO* IN ISRAEL

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Abstract

Since spring 1998, a new disease causing high mortality has afflicted cultured ornamental and common carps *(Cyprinus carpio)* in Israel. The clinical signs of the disease are fatigue, gasping movements in shallow water, gill necrosis, sunken eyes, pale patches on the skin and increased mucus secretion. The disease was experimentally transmitted to koi and common carp of various ages by injection with affected tissue filtrates, cohabitation trials and exposure to the isolated viral agent. Immersion of healthy fish in tanks containing 2.7×10^4 plaque forming units (PFU) per l or intraperitoneal injection (0.2 ml) of a viral inoculum of 1000 PFU/ml were sufficient to induce the terminal disease in over 80% of the fish. Here we report that the disease is restricted to *C. carpio,* that young fish of 2.5 g and 6 g are more sensitive to the virus than adults (230 g) and that the virus remains active in the water for at least four hours.

Introduction

Mass mortality of cultured common carp and koi *(Cyprinus carpio)* was recorded in Israel during the spring of 1998. The first two affected carp farms were located on the Carmel shore, but during the following three years the disease spread to 90% of all carp farms, from northern Israel to the southern center (Fig 1). Since 1998 similar outbreaks occurred every

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Fig. 1. The spread of the carp viral disease in Israel between 1998 and 2000. Area of infected farms —— in 1998, ... in 1999 and - - - - in 2000.

spring and fall, as soon as the water temperature reached 22-26°C. The mortality rate in the affected ponds was 80-90% in fry as well as in older, market-size fish.

This disease caused great economic losses to the food and ornamental carp industry in Israel, and severe difficulties in marketing these expensive and magnificent fish around the world. At the end of 1998 the loss in Israel was estimated as $1.2 million for the common carp industry and $0.8 million in ornamental carp exports. Despite intensive efforts to decrease the damage during the last four years, the carp disease has already cost Israeli aquaculture $3 million yearly. A similar carp disease has been reported from the USA, several European countries (Germany, Britain and the Netherlands), Indonesia, Korea and Japan (Walster, 1999; Hedrick et al., 2000; Miyakazi et al., 2000; Oh et al., 2001), causing severe losses to fish breeders, retailers and hobbyists the world over. Restrictions on the transport of carps and quarantines have been applied to halt the spread of the virus.

Our clinical studies for Spring Viremia of Carp disease (SVC), as well as that of other known viruses such as Carp Herpes Virus (CHV), Viral Hemorrhagic Septicemia (VHS), Epizootic Hemopoetic Necrosis (EHN) and other salmonoid viruses, were negative, suggesting the involvement of an as yet unclassified virus. In this study we used three methods to induce the disease in fish: injection of organ filtrates, cohabitation with sick fish, and exposure by injection or immersion to a virus harvested from tissue culture.

Materials and Methods

Pathological and parasitological examination. A number of symptomatic carp and koi fish were collected from three different farms and transferred live to the Central Fish Health Laboratory in Nir David. The fish were subjected to parasitological and microbiological examinations. Brain heart infusion agar (BHIA) and tryptic soy agar (TSA; Difco) were used to isolate bacteria from the gill, spleen, liver and kidney. These organs were also screened for pathogenic viruses at the Kimron Veterinary Institute, Beit Dagan, Israel.

Histological study and electron microscopy. Tissue samples of the fish organs were fixed in 10% neutral buffered formaldehyde, and then processed using routine histological techniques. Tissue sections were stained with hematoxylin and eosin.

Samples taken from the same organs were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at 4°C. The tissue was then fixed for one hour in 2% OsO_4, dehydrated in graded ethanol series and embedded in agar 100 resin blocks. Semi-thin sections were stained with toludine blue. Ultra-thin sections were cut on an ultratome III (LKB, Bromma, Sweden) with a diamond knife. Uranyl acetate and lead citrate stained sections were mounted on copper grids and examined by Jeol 100CX II transmission electron microscope at the National Center for Mariculture, Eilat, Israel.

Injection with fish tissue extracts. Homogenates were prepared from the brain and kidney of five diseased koi and filtrated through a 100 µm filter. Half of the homogenate volume was then refiltered through a 0.22 µm filter and the filtrates were used for injection. Groups of 11 naïve common carp (300 g) were injected intraperitoneally with 0.4 ml of homogenates or filtrates. Phosphate saline buffer (PBS) was administered to the control fish.

Selection of sick fish for disease generation. Symptomatic fish were collected and transferred to two 500 l tanks at the Dor Research Station. The tanks were divided into four chambers by plastic nets and maintained at a water temperature of 24°C. The sick fish were placed in one of the chambers and the adjoining chamber was stocked with a group of uninfected fish. Every five days, another group of naïve fish was added to the tanks and placed in the next chamber, in a circulatory manner, so that there was always a supply of sick fish in the tanks. The fish were routinely examined for parasites and bacterial pathogens. Dead fish were removed daily. Fish were used on their fifth day of exposure to generate the disease in the cohabitation experiments of this study.

Transmission to other species. To see whether the disease could be transmitted to other cyprinoids and species, tilapia *(Oreochromis niloticus)*, silver perch *(Bidyanus bidyanus)*, silver carp *(Hypophthalmichthys molitrix)*, goldfish *(Carassius aurata)*, black carp *(Ctenopharyngodon idella)* and common carp *(C. carpio)* were stocked together with infected carps. Ninety fish of each species were placed in three 500 l tanks (30 fish per tank), supplied with fresh water (0.9 l/min) at a temperature of 23-25°C. Cohabitation was effected by exposure to five infected fish per tank for five days. After the exposure period, the infected fish were removed and the tanks were washed with fresh water for 48 hours. To test whether fish of the resistant species could transmit the virus, we exposed 30 naïve carp to fish of the above species which had cohabited with the sick carp.

Susceptibility of common carps as a function of age. Naïve common carp of different weights and ages (2.5, 6 and 230 g) were challenged. For each weight group, 100 fish were placed in a 500 l tank supplied with fresh water (0.9 l/min) at a temperature of 23-25°C. Each group was exposed to infected fish by cohabitation with five infected fish for five days. Fifty unexposed fish of each weight group were used as the negative control. Fish were observed for 21 days for mortality and clinical signs.

Virus propagation. Cell culture preparation, virus propagation and titration are described by Hasegawa et al. (1997), Neukirch et al. (1999) and Hutoran et al. (submitted). Briefly, to propagate the virus, carp fin cell cultures were maintained in 75 ml flasks (Nunc) at 22°C in 60% Dulbecco's modified eagle medium, 20% Leibovitz L-15 medium, 10% fetal calf serum and 10% tryptose phosphate, supplemented with 1% N-[2-Hydroxyethel]piperazine-N-[2-ethane-sulfonic acid] (HEPES) and antibiotics. The cells were infected with 1.5 ml kidney extract from infected fish and diluted 1:10 with the culture medium. Following the appearance of plaques, the medium and the cells were harvested and stored at -70°C until use. The virus titter in each harvest was determined by standard plaque assay.

The infectivity of viruses harvested from tissue cultures. The cell cultures were used to assess whether the isolated virus induces the disease and to determine how long the virus remains infective in water. Ninety naïve 10 g common carps were injected intraperitoneally

with 0.2 ml containing 1000 PFU/ml of virus harvested from tissue culture. Three other groups (n = 30) were immersed for 40 min in a 10 l bath, containing 2.7 x 10^4 PFU/l. The first of these three groups (T0) was immersed immediately following addition of the virus to the water, the second group (T4) four hours after and the third (T21) 21 hours after addition of the virus to the water. In addition, a group of 60 fish was injected intraperitoneally with 0.2 ml of PBS as a control.

Results

Clinical and pathological signs. During this study, fish surveyed in over 30 farms exhibited fatigue and gasping movements in shallow water. External clinical signs included sunken eyes, gill necrosis, pale skin patches due to lack of mucus secretion during the initial stages of the disease, and increased secretion during the later stages. Internal examination revealed an enlarged liver, necrotic hemopoetic cells in the kidney and enlarged tubules. Diseased fish were characterized by a greater susceptibility to nonspecific parasites such as *Ichthyobodo necatix, Ichtyophthirius, Argulus* spp*., Cryptobia* spp*., Trichodina* spp. and gill Monogenea. Nonspecific bacterial pathogens such as *Aeromonas hydrophila* and *Myxobacteria* were found sporadically, excluding the possibility of bacterial infection as a primary cause of fish death.

Histological study. Prominent pathological changes were observed in tissues of infected fish. The most significant lesions were in the gill and kidney. Severe hyperplasia, lamellar fusion and adhesion of gill filament were common (Fig 2a). Foci of necrosis and necrotic cells were observed in the liver (Fig 2b) and nuclear inclusions were present in nuclei of the renal glomerulae (Fig 2c).

Electron microscopy. Virus-like particles were detected in fixed intestinal tissues of sick fish, near the nuclear membrane (not shown). Their core diameter was approximately 120 nm, with enveloped hexagonally shaped nucleocapsids (Fig 3).

Injection with fish tissue extracts. Mortality among healthy fish injected with tissue homogenates and filtrates from diseased koi carp was 37% and 82%, respectively, 7-10 days after injection, whereas there was no mortality amongst saline-injected fish. These results strongly suggest that the carp disease is virus borne, as demonstrated by Hedrick et al. (2000), Gray et al. (2002), Gilad et al. (2002), Body et al. (2000), Neukirch et al. (1999) and Hutoran et al. (submitted).

Transmission to other species. In the cohabitation trial, 72% of the common carps died from the disease eight days after exposure, while fish of other species survived the treatment. When healthy carp were exposed to fish that had cohabited with sick carp, only common carp transmitted the virus, causing 68% mortality among the healthy carps; fish of the resistant strains remained alive and did not infect the naïve carps.

Susceptibility of common carps as a function of age. In the age test, ten days following exposure to the sick fish, 92.5% of the 2.5 g fish died (Fig. 4). Similar results were recorded for 6 g fish. On the other hand, 230 g fish were more resistant and only 56% died. Mortality of fish of all sizes started 5-7 days and peaked 10-14 days after exposure, while none of the control fish died during the experiment. These results clearly show that young, small fish are more susceptible to the disease than adults.

The infectivity of viruses harvested from tissue culture. Fig. 5 shows that 99-100% of the fish injected with the virus harvested from the tissue culture or immersed immediately (T0) after the virus from the tissue culture was added to the bath died within 14 days after infection, while all the PBS-injected fish remained alive. The virus in the water remained infectious for four hours, but not for 21. We therefore conclude that the virus is transferred via water, where it remains active for at least four hours.

Discussion

Massive mortality of carps has been observed among food and ornamental carp (*C. carpio)* in many fish farms throughout Israel. A similar disease was also reported in North America, Europe, Indonesia, Korea and Japan, causing severe losses to the fish industry (Walster,

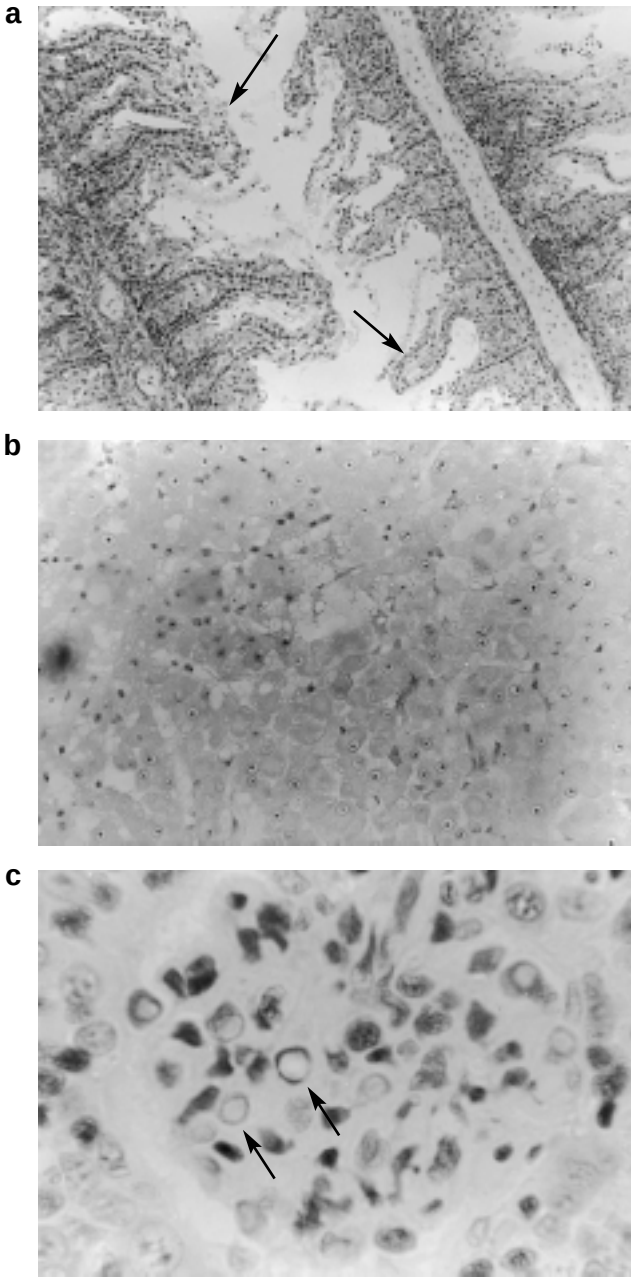

Fig. 2. (a) Histological section (x 200) of the gill. The small arrow indicates hypertrophy of epithelial cells and fusion of secondary lamellae of the gill. The big arrow points at a lifting of the epithelium from the underlying vascular spaces in the gill and necrosis of individual cells. (b) Histological section (x 400) of the liver. The liver parenchyma contains foci of necrosis, degeneration and loss of hepatic architecture. (c) Histological section (x 1000) of the kidney. Nuclear changes in the kidney lesion and inclusion bodies (arrows) are evident in the nuclei of the capillaries in the glomerulae.

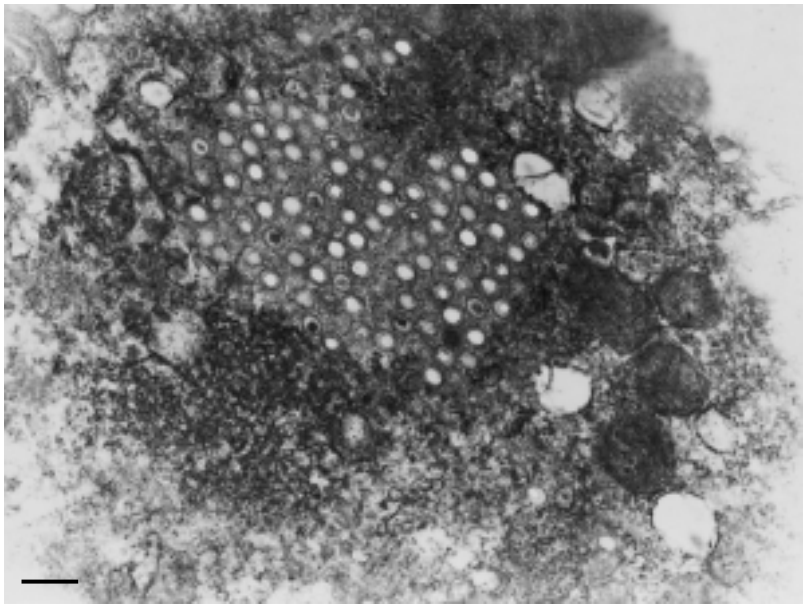

Fig. 3. Electron micrograph of virus-like particles from the intestinal tissue of symptomatic common carp (scale bar = 300 nm).

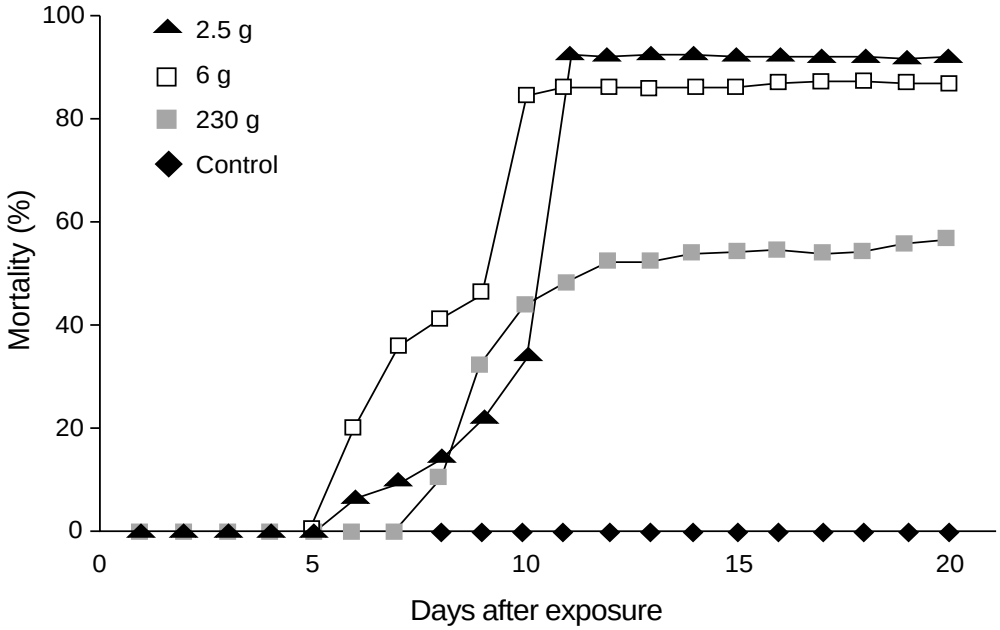

Fig. 4. Mortality rate (n = 100 fish in each group) among fish of different size groups following cohabitation with sick fish. Adult carp (230 g), 3-month old carp (6 g), and 1-month old carp (2.5 g) were exposed to the disease by cohabitation with sick fish for five days. Fifty unexposed fish of each size were used as a control.

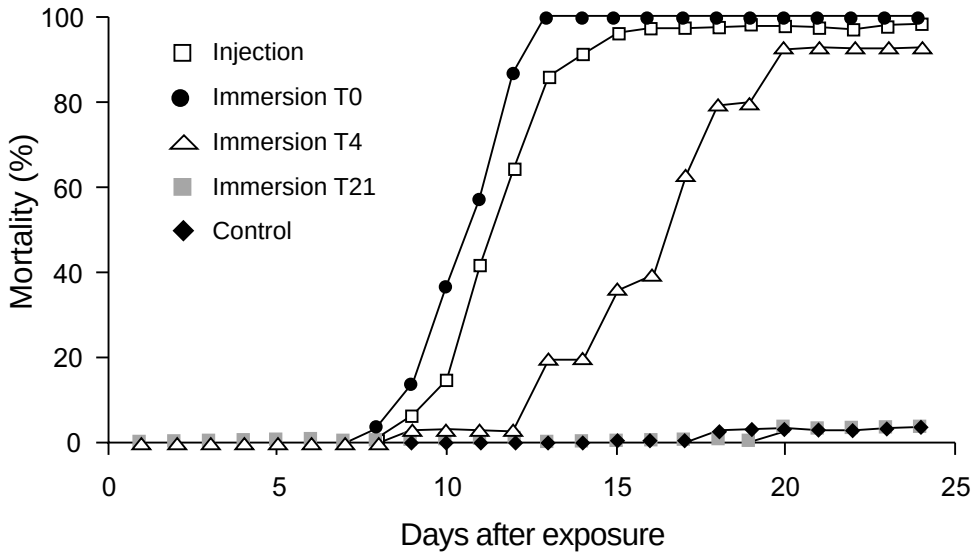

Fig. 5. Mortality rate of carp injected with virus harvested from infected cell cultures (n = 90, avg wt = 10 g) or immersed immediately (T0), four hours (T4) or 21 hours (T21) after addition of the virus to the water (n = 30 fish in each group, avg wt = 10 g). Sixty fish were injected with PBS as a control.

1999; Hedrick et al., 2000; Miyakazi et al., 2000; Oh et al., 2001). The carp disease is highly infectious both in aquaculture and under experimental conditions and restricted to a water temperature of 22-26°C. Thus, the disease erupts at transient seasons. The major external sign observed in affected fish was severe gill necrosis. Histological and electron microscope examinations revealed the systemic nature of the disease. Intranuclear inclusions and tissue degeneration were observed in kidney and liver cells. Virus particles detected in intestinal tissue have a diameter of 120 nm and morphologically resemble those of 110 nm and 100 nm that were isolated from the gill by Hedrick et al. (2000) and Bretzinger et al. (1999), respectively.

In this study we used three methods to induce the disease in fish: injection of organ filtrates, cohabitation with sick fish and exposure by injection or immersion to a virus harvested from tissue culture. The induced and spontaneous disease had a typical epidemic progression and kinetic: lack of symptoms during the first five days; mortality beginning after 6-8 days, reaching a plateau 10-12 days post-exposure.

Here we demonstrate that the virus remains infective in water for at least four hours, explaining the highly contagious nature of the virus in the ponds. The findings that the virus is stable and transmitted via the water were used by Ronen et al. (submitted) to immunize fish by spreading an isolated attenuated virus in tanks.

Oh et al. (2001) reported that the disease affected fish of various ages. However, the results of our cohabitation trial showed greater susceptibility of fry (1-3 months, 2.5-6 g) than of mature fish (over a year, 230 g), demonstrated by mortality of over 85% and 56%, respectively. We speculate that due to the immature immune system of young fish, they are more susceptible to the virus.

We evaluated the susceptibility of economically important fish species *(H. molitrix, C. idella* and *C. aurata)* in the same

Cyprinidae family, and their ability to function as virus carriers. We found that the disease is restricted to *C. carpio* and did not even affect *C. aurata,* which belongs to the same subfamily. This virus has a very narrow host range. We believe that the virus causing the disease subsisted in nature. Upon invasion of the intensively cultured carp population, it adapted and genetically diverged and, thus, is restricted to this species. Which are the viral gene(s) that confine the virus to carp fish cells, what is the restrictive stage in the virus propagation and what are the cellular factor(s) that allow the productive infection, these are questions still to be resolved.

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